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Role of the *ZWILLE* gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis

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Postembryonic development in higher plants is marked by repetitive organ formation via a self-perpetuating stem cell system, the shoot meristem. Organs are initiated at the shoot meristem periphery, while a central zone harbors the stem cells. Here we show by genetic and molecular analyses that the *ZWILLE* (*ZLL*) gene is specifically required to establish the central–peripheral organization of the embryo apex and that this step is critical for shoot meristem self-perpetuation. *zll* mutants correctly initiate expression of the shoot meristem-specific gene *SHOOT MERISTEMLESS* in early embryos, but fail to regulate its spatial expression pattern at later embryo stages and initiate differentiated structures in place of stem cells. We isolated the *ZLL* gene by map-based cloning. It encodes a novel protein, and related sequences are highly conserved in multicellular plants and animals but are absent from bacteria and yeast. We propose that *ZLL* relays positional information required to maintain stem cells of the developing shoot meristem in an undifferentiated state during the transition from embryonic development to repetitive postembryonic organ formation.

Keywords: *Arabidopsis* embryo/cell fate/self-perpetuation/shoot meristem/stem cells

Introduction

Stem cells in the center of the shoot meristem are the ultimate source from which all tissues of the growing shoot are derived (Barlow, 1978; Clark, 1997; Meyerowitz, 1997; Laux and Mayer, 1998). They are considered undifferentiated in the sense that they lack morphological features associated with cells in mature tissue, such as a large central vacuole. Clonal analyses suggest that the pluripotent shoot meristem stem cells are specified in response to positional cues (Ruth *et al.*, 1985). Differentiating daughter cells enter specific developmental pathways according to their positions and are incorporated into organ primordia in the peripheral zone.

Several genes have been shown to regulate the maintenance of the stem cell population and thus the indeterminate nature of the shoot meristem itself. The first specific regulator identified was the maize *KNOTTED1* (*KN1*) gene, which encodes a homeodomain protein that promotes meristem cell fate in a non-cell-autonomous manner (Vollbrecht *et al.*, 1991; Smith *et al.*, 1992; Sinha *et al.*, 1993). The related *SHOOT MERISTEMLESS* (*STM*) gene from *Arabidopsis* is required to prevent stem cells from being incorporated into organ primordia, and thus from differentiating (Clark *et al.*, 1996; Endrizzi *et al.*, 1996; Long *et al.*, 1996). The *WUSCHEL* (*WUS*) gene is necessary for cell identity in the meristem center and *wus* mutations lead to termination of meristem activity (Laux *et al.*, 1996). While *WUS* and *STM* are required for shoot and floral meristem activity throughout development, mutations in the *TERMINAL FLOWER* gene specifically result in the conversion of the inflorescence meristem center into determinate flowers (Alvarez *et al.*, 1992). The *CLAVATA* (*CLV*) genes promote organ formation and/or regulate meristem cell proliferation and appear to have antagonistic roles to the genes mentioned above (Clark, 1997). *STM* and *CLV1* seem to competitively regulate the balance between undifferentiated cells and organ formation in response to positional information (Clark *et al.*, 1996; Laux and Schoof, 1997). *CLV1* encodes a putative membrane-bound receptor kinase, suggesting a function in a signaling pathway (Clark *et al.*, 1997). Genetic analysis suggests that *WUS* is a putative target for *STM* and *CLV1* regulation (Endrizzi *et al.*, 1996; Laux *et al.*, 1996).

Development of the primary shoot meristem is initiated during the establishment of the basic body organization of the embryo (Laux and Jürgens, 1997) as visualized by transcriptional activation of the *STM* gene in central apical cells of the globular embryo (Long *et al.*, 1996). Histological analyses of organ initiation (Spurr, 1949; Kaplan, 1969) and the analogous defects of cotyledon and leaf formation in the *stm* mutant (Endrizzi *et al.*, 1996) suggest that at least some similar mechanisms are involved during the initiation of cotyledons at the globular embryo stage, and leaf initiation in postembryonic shoot meristems. However, there are also differences suggesting that the apical domain of the globular embryo is not fully equivalent to a shoot meristem (Laux and Mayer, 1998). For example, the initiation of leaves but not cotyledons is affected by mutations in the *WUS* (Laux *et al.*, 1996) or *ZLL* genes (Jürgens *et al.*, 1994; Endrizzi *et al.*, 1996). While recent genetic and molecular analyses have provided models for the way in which the shoot meristem is maintained (Clark, 1997; Meyerowitz, 1997; Laux and Mayer, 1998), little is known about how the organization and regulatory networks that govern shoot meristem self-perpetuation are established during embryogenesis. Here we report the isolation of the *ZLL* gene and an analysis of its role. Functional analyses of *zll* mutants and gene expression data both indicate that *ZLL* is required for stem cell fate within the developing embryonic shoot meristem.

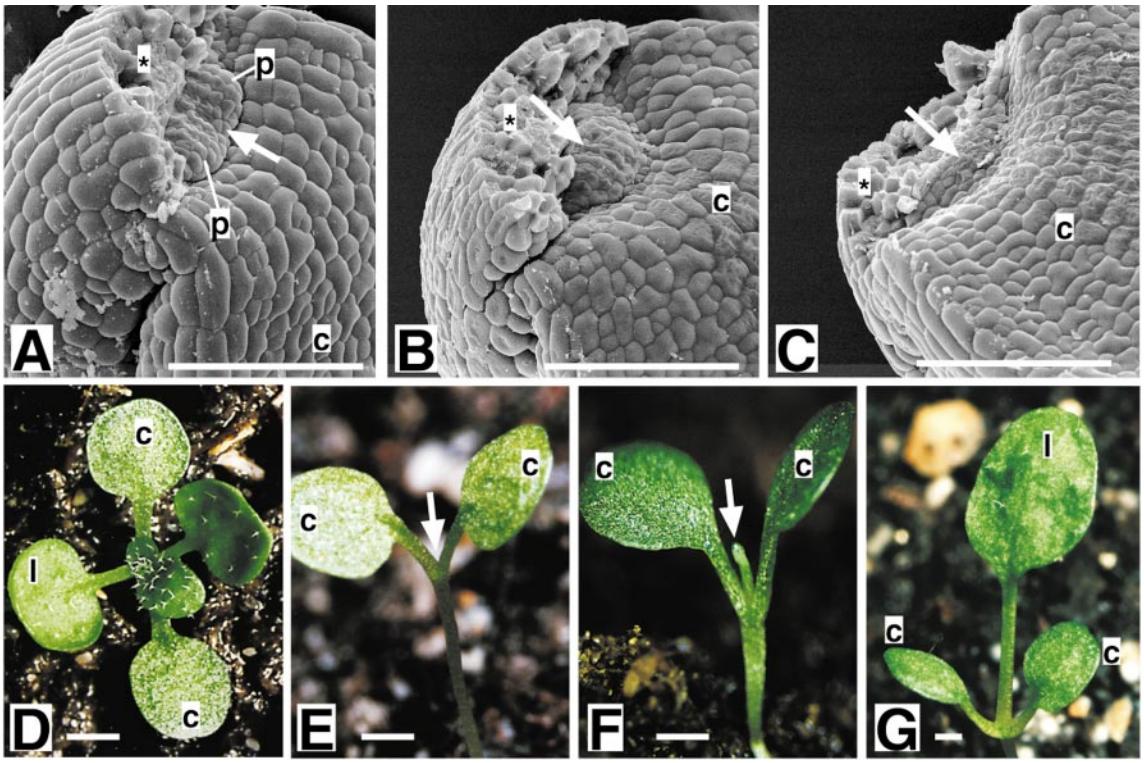

Fig. 1. Embryonic and seedling phenotype of *zll* mutants. (A–C) Scanning electron microscopy of mature embryo apices where one cotyledon has been removed (★). (**A**) In wild-type, the shoot meristem (arrow) has initiated the first two leaf primordia, p. In *zll* embryos, either a single bulge (**B**, arrow) or a flat apex (**C**, arrow) has formed instead of a shoot meristem. (D–G) 10-day-old seedlings. (**D**) In wild-type, the shoot meristem has initiated a rosette of leaves, l. *zll* seedlings either display an empty apex (**E**, arrow), a filamentous structure (**F**, arrow) or a solitary leaf (**G**, l) instead of a shoot meristem. c, cotyledon. Each bar represents 50 μm in A–C, 1 mm in D–G.

Results

In order to study early events in shoot meristem development, we analyzed 16 EMS (ethyl methanesulfonate)-induced mutant alleles of the *ZLL* gene (Jürgens *et al.*, 1994). All *zll* mutants showed indistinguishable seedling phenotypes that suggested a defect in embryonic shoot meristem establishment. Complementation analyses between *zll*-3 and the similar mutant *pinhead* (*pnh*) (McConnell and Barton, 1995) demonstrated that the two mutations are allelic (data not shown). Unless stated otherwise, we used *zll*-3 for our analyses.

zll embryos form differentiated structures instead of a shoot meristem

In mature wild-type embryos, the shoot meristem can be recognized as a ridge of cells, five to six cells long and two cells wide (Figure 1A), flanked by the first two true leaf primordia which have arisen from it. In contrast, mature *zll* embryos displayed a single bulge of cells, three to nine cells across, spanning the whole apex (Figure 1B). In some cases, the cell number was reduced and the shape of the apex was flat (Figure 1C).

After germination, the shoot meristem in wild-type seedlings continually initiates new organs and gives rise to a rosette of leaves (Figure 1D). *zll* seedlings, in contrast, showed an 'empty', flat apex (Figures 1E and 2A), solitary filamentous structures (Figure 1F) or leaves (Figure 1G) instead of a shoot meristem. A similar range of seedling phenotypes has been described for the *pnh* mutant (McConnell and Barton, 1995). In *zll* seedlings, the orientation of solitary leaves relative to the cotyledons was variable, in contrast with wild-type. Thus, no regular wild-type leaf was formed, indicating that *zll* shoot meristem development was disrupted before the initiation of the first true leaves. Infrequently, intermediate forms between filamentous structures and leaves, radially symmetric or partially fused leaves, were observed (data not shown). The number of seedlings with central structures correlated with the number of embryos with bulged apices, suggesting that an apical bulge represents a central structure primordium. All other parts of the seedling, such as the cotyledons, shoot axis and the root meristem, were not visibly affected in *zll* mutants, suggesting that *ZLL* function is required specifically in the embryo for shoot meristem development.

zll mutations affect developmental decisions in the embryo apex

Occasionally, individual *zll* embryos initiated two leaf primordia at the periphery of the apex as in wild-type embryos. In such 'escapes', a functional primary shoot meristem was formed, in contrast with *zll* seedlings displaying the range of phenotypes described above (Table I). Additional observations suggested that different *zll* alleles, although they produced qualitatively indistinguishable seedling defects, differed in the frequency of escape seedlings. The reason for this quantitative difference is unclear, but the parental genotype did not play a role and reciprocal crosses between pairs of *zll* alleles resulted in transheterozygous progenies that displayed an

Table I. Primary shoot meristem formation in *zll* embryos

	zll seedling phenotypes			
	E	F	L	2L
Seedlings (n)	126	180	68	63
PSM (%)	0	0	0	98

In *zll* seedlings that displayed an empty apex (E), a central filamentous structure (F), a solitary leaf (L) or at least two leaves (2L), the percentage of plants that showed a primary shoot meristem (PSM) is given.

escape frequency similar to or intermediate between their parents, suggesting that different alleles reflect different doses of *ZLL* activity (data not shown).

zll seedlings form adventitious shoot meristems

Although *zll* mutants were defective in embryonic shoot meristem development, they could initiate *de novo* adventitious shoot meristems (ASM) postembryonically in the axils of the cotyledons (Figure 2B–F). In seedlings with either a flat apex or with a radially symmetric central structure, ASMs were formed on average 10 days after germination in both cotyledonary axils (Figure 2C–E). In contrast, in seedlings with a bilaterally symmetric central leaf, ASMs were initiated 5 days later on average and were restricted to the cotyledonary axil facing the upper side of the leaf (data not shown). Histological analyses and staining of nuclei showed that ASMs were positioned at the base of cotyledonary petioles (Figure 2D–E), indicating that the ASMs were not simply lagging primary shoot meristems. *zll* ASMs correctly initiated leaves at the periphery, but compared with wild-type shoot meristems they were larger and elongated (Figure 2F and G). This resulted in fasciated shoots giving rise to leaves and floral meristems in an abnormal arrangement (Figure 2G). Occasionally, *zll* adventitious shoots did not initiate meristems in the axils of cauline leaves (Figure 2I), similar to the *pnh* mutant (McConnell and Barton, 1995). However, we did not observe this defect in the primary shoots of escape *zll* plants, suggesting that it is a secondary effect of the fasciated shoot architecture.

Thus, *ZLL* seems not to be necessary for shoot meristem formation or function *per se*, but to be specifically required for establishing a functional primary shoot meristem in the embryo.

ZLL is required to prevent cell differentiation in the center of the embryonic apex

In order to study the cellular basis of the *zll* defect, we analyzed embryo and seedling development at the histological level. During early stages of embryogenesis (globular stage to torpedo stage) no differences were found between wild-type and *zll* (data not shown). In median sections the shoot meristem can be recognized as a group of small, densely staining cells with large nuclei, which are located between the cotyledons in the mature embryo (Figure 3A) and between the leaf primordia in the seedling (Figure 3B). The surrounding differentiated cells are larger and stain less densely, and their central vacuole occupies most of the cell lumen.

In contrast, we did not detect meristem cells in mature *zll* embryos or in *zll* seedlings. The apices of *zll* embryos were variably shaped, and the cells had neither prominent nuclei, nor did they stain more intensively than surrounding differentiated cells (Figure 3C). In *zll* seedlings, the cells in the apex were larger and more vacuolated than wild-type meristem cells, suggesting that these cells had initiated differentiation (Figure 3D). In contrast with the shoot meristem, root meristem organization appeared to be normal in *zll* mutants (data not shown), suggesting that the two meristems are regulated by different processes.

ZLL regulates the spatial pattern of STM expression late in embryogenesis

In order to determine the stage at which *ZLL* is required, we analyzed the expression of *STM* as a molecular marker for shoot meristem cell identity in *zll* embryos compared with wild-type. *STM* expression is confined to central apical cells of wild-type embryos from the globular stage onwards and is downregulated in the organ anlagen at the periphery of the shoot meristem, but maintained in the center, during postembryonic development (Long *et al.*, 1996).

STM expression appeared not to be affected in *zll* mutants during early stages of embryogenesis (Figure 4A, B, E and F), suggesting that the shoot meristem program was initiated correctly. However, at later embryo stages the spatial expression pattern of *STM* was markedly altered: expression was downregulated in the center of the apex (Figure 4G), in contrast with wild-type (Figure 4C), and became confined to a small group of lateral cells. This downregulation of central *STM* expression is consistent with the differentiation of the *zll* embryo apex and may explain why *zll* seedlings occasionally mimic a weak *stm* phenotype (Endrizzi *et al.*, 1996) and produce fused leaves at the position of the shoot meristem. *STM* expression appeared to be normal in *zll* mutants during postembryonic development (Figure 4D and H), as expected from the mutant phenotype.

Differentiation of central apical cells in zll embryos is not affected by clv mutations

CLV and *STM* antagonistically regulate the balance between undifferentiated cells and cell differentiation in shoot and floral meristems, with *CLV* possibly promoting organ formation at the periphery and *STM* promoting meristem cell fate in the center (Clark *et al.*, 1993, 1996). Our observation that *STM* expression in *zll* embryos was downregulated at the apex center raised the possibility that an increased *CLV*/*STM* ratio caused ectopic differentiation in *zll*. We therefore analyzed *zll clv* double mutants to determine whether CLV activity was required for ectopic cell differentiation in *zll* apices.

zll clv1 embryos displayed the *zll* single mutant defects, such as flat apices (Figure 5B) or solitary central organs, suggesting that *CLV1* is not required for differentiation of central apical cells in *zll* embryos. This observation is consistent with findings that *CLV* mutations cannot rescue embryonic shoot meristem development in *stm* (Clark *et al.*, 1996). Adventitious shoot meristems in *zll clv1* double mutants were drastically increased in size by the *clv1* mutation (Figure 5C), as expected from the enlarged meristem in *clv1* single mutants. Double mutant flowers had similar but more variable organ numbers compared

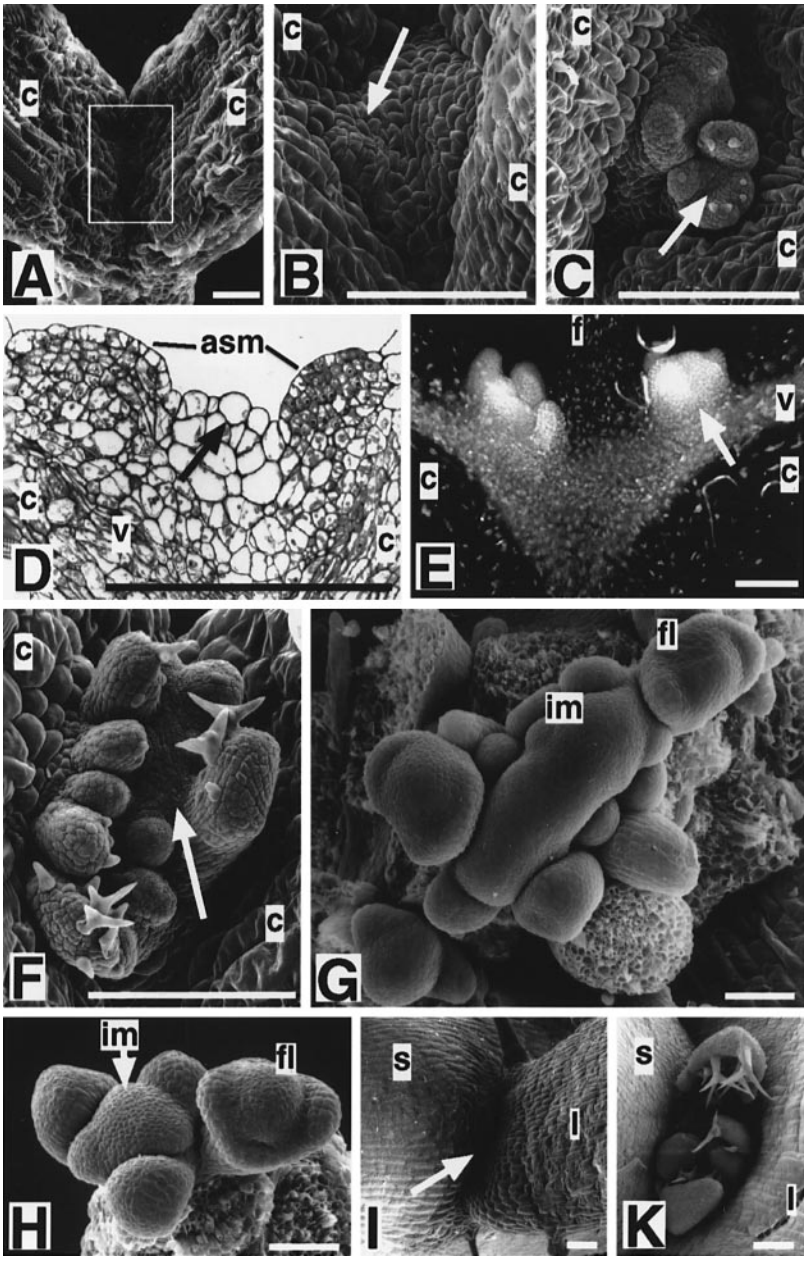

Fig. 2. Postembryonic *zll* development. (A–C, F–K) Scanning electron microscopy images, (A–G and I) *zll*, (H and K) wild-type. *zll* seedlings lacking a primary shoot meristem (**A**) initiate adventitious shoot meristems at the base of the cotyledons [(**B**) and (**C**), arrows; range corresponds to rectangle in (A)]. (**D**) In longitudinal median sections the differentiated central apical cells are still present (arrow) after initiation of ASMs (asm). (**E**) DAPI-stained seedlings suggest that ASMs (arrow) were initiated at the petiole of the cotyledons. (**F**) The elongated ASM (arrow) has initiated supernumerary leaf primordia at its periphery. (**G**) The *zll* inflorescence meristem, im, is fasciated and gives rise to supernumerary floral meristems, fl. (**H**) Wild-type comparison with (G). (**I**) Occasionally, *zll* adventitious fasciated shoots fail to initiate axillary shoot meristems (arrow). (**K**) Wild-type comparison with (I). c, cotyledon; f, filamentous structure; l, leaf; s, stem; v, vasculature. Each bar represents 50 μm.

with *clv1* (Table II). The same phenotypes as for *zll clv1* were observed for *zll clv3*, consistent with the notion that *CLV1* and *CLV3* act in the same process (Clark *et al.*, 1995).

Map-based cloning of the ZLL gene

The *ZLL* gene was initially mapped with respect to morphological markers and was found to be linked to the *TT3* gene on chromosome 5 (data not shown). To isolate the *ZLL* gene, we used RFLP and PCR-based mapping of recombination breakpoints in about 2500 meiotic F1 events from the cross *zll* (L*er*)×wild-type (Nd). We isolated ~1 Mb of contiguous genomic DNA from yeast artificial chromosome (YAC) libraries (Creusot *et al.*, 1995), spanning the region between the *TT3* and the *CRA1* genes. We initiated chromosome walks from both directions with bacterial artificial chromosome (BAC) and P1 clones (Liu *et al.*, 1995) and eventually localized *ZLL* on a single BAC clone of ~100 kb, CO59 (Figure 6A). From this BAC we constructed a cosmid library, established a cosmid contig and narrowed down the position of the gene to a region of ~30 kb in which we did not find any recombination. Using the cosmid clone c3 (Figure 6A), we isolated eight cDNA clones that represented a single transcript. Two independent cDNA

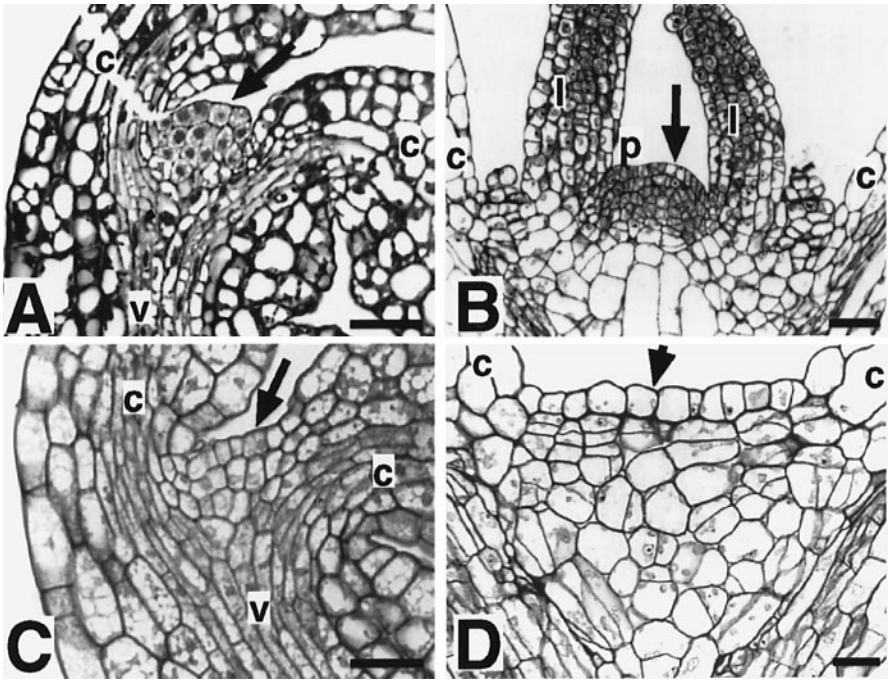

Fig. 3. Cell types are altered in *zll* apices. 1 μm longitudinal median sections of mature embryo (A and C) or seedling (B and D) apices. In wild-type (**A** and **B**), the shoot meristem displays small, densely staining cells (arrows). Apical cells in *zll* mutants (**C** and **D**) appear to have initiated differentiation (arrows); they are larger and stain less densely than the corresponding wild-type cells. c, cotyledon; l, leaf; p, primordium; v, vasculature. Each bar represents 20 μm.

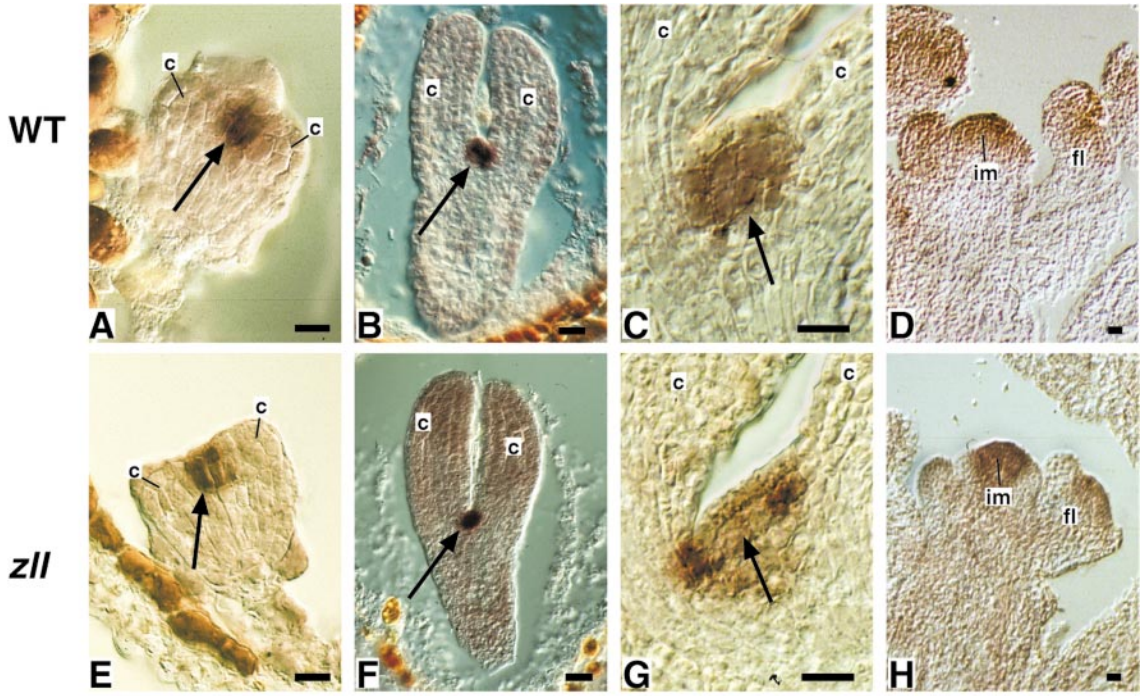

Fig. 4. Expression patterns of *STM* in wild-type and *zll* mutants. *In situ* hybridization of longitudinal median sections of wild-type (A–D) and *zll* (E–H) with *STM* antisense RNA. (**A** and **E**) early heart stage embryo; (**B** and **F**) torpedo stage embryo. *STM* expression (arrows) is confined to apical cells between the cotyledonary primordia, c, both in wild-type (A and B) and in *zll* (E and F) embryos. (**C** and **G**) Bent cotyledon stage embryo. In wild-type (C), *STM* is expressed in a single group in the center of the shoot meristem primordium (arrow). In *zll* embryos (G) the expression is reduced in the central part of the apex (arrow), but instead is present in two patches close to the axils of the cotyledons, c. (**D** and **H**) Inflorescences. *STM* is expressed in the center of both the inflorescence meristem, im, and floral primordia, fl, in wild-type (D) and in *zll* (H). Tissue was collected, fixed and processed under the same conditions. Each bar represents 10 μm.

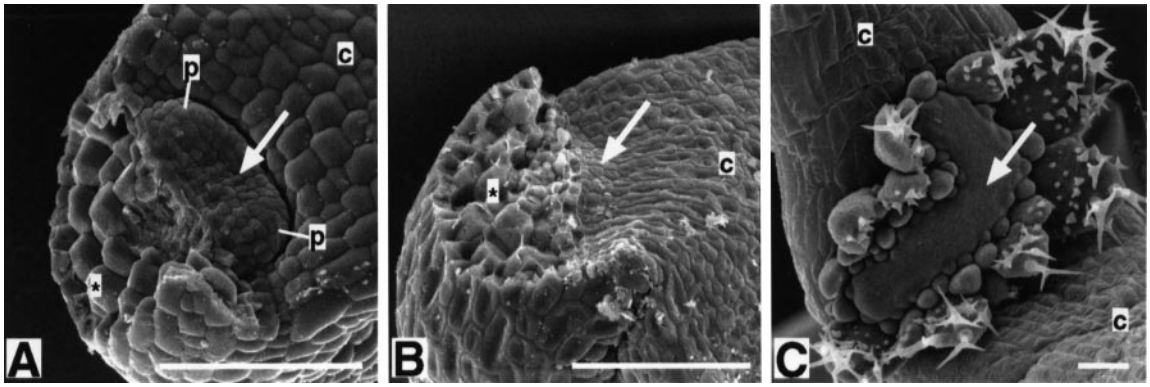

Fig. 5. *zll clv* double mutants. (A and B) Scanning electron microscopy of mature embryo apices where one cotyledon has been removed (★).(**A**) In *clv1* a shoot meristem (arrow) and two leaf primordia, p, are present (compare with wild-type, Figure 1A). (**B**) *zll clv1* double mutant embryos lack a shoot meristem (arrow; compare with *zll*, Figure 1B and C). (**C**) Scanning electron microscopy of a *zll clv1* seedling apex. The adventitious meristem (arrow) is enlarged compared with *zll* (Figure 2F). c, cotyledon. Each bar represents 50 μm.

clones of ~3.3 kb that were isolated from different libraries both displayed an open reading frame of 2964 nucleotides. The presence of stop codons upstream of this open reading frame indicates that these cDNAs contain the full coding region. We confirmed that this reading frame represented the *ZLL* gene by identifying mutations in the cognate genomic DNA of eight different *ZLL* alleles (Figure 6B), seven of which represented G to A changes, typical for mutations induced by EMS, while one (*zll*-15) was an insertion of a single A.

ZLL is a member of a novel gene family specific to multicellular organisms

The *ZLL* gene encodes a protein of 988 amino acids (Figure 6B). The predicted protein is hydrophilic, suggesting that ZLL is soluble in the cytosol. Its N-terminal region (residues 1–123) is highly proline rich (14%) and does not show significant similarities with known sequences. In contrast, the protein sequence from residue 124 to 988 shows 75% identity with the *Arabidopsis* ARGONAUTE1 (AGO1) protein (DDBJ/EMBL/GenBank accession no. U91995) and 43% identity with hypothetical proteins from *Caenorhabditis elegans* (e.g. DDBJ/EMBL/GenBank accession no. Z69661; Figure 6C). In addition, very high similarities between ZLL and sequences from plants, vertebrates and invertebrates were found at the C-terminus (Figure 6D). The four stop-mutations identified suggest that this part of the protein is not present in the respective *zll* alleles (Figure 6B). Except for ZLL and AGO1, the latter of which is required for leaf development (Bohmert *et al.*, 1998), all other members of this sequence group are derived from genome or random cDNA sequencing projects and their functions are unknown. We did not identify sequence motifs suggestive of known functional domains in ZLL. Missense mutations affect residues that are identical in all available sequences (Figure 6C), suggesting that these residues are essential for a general function of this class of putative proteins. Interestingly, we did not find any *ZLL*-related sequences from bacteria or budding yeast for which the complete genome sequences are known, indicating that *ZLL* and other members of the gene family play roles specific to a multicellular context.

Expression of ZLL

We used *in situ* hybridization to analyze the *ZLL* expression pattern. *ZLL* mRNA was found in provascular cells at all stages of development (Figure 7A–E). Postembryonically, *ZLL* expression in provascular cells seemed to presage organ initiation (Figure 7C). Cross sections of embryo axes showed that *ZLL* expression was restricted to small groups of cells, presumably at the phloem pole (Figure 7E). In addition to provascular cells, *ZLL* mRNA was detected in the apex late in embryogenesis (Figure 7D) at about the time when *zll* apical development deviates from wild-type. *ZLL* mRNA, as a molecular marker for *ZLL* expressing cells, was distributed in provascular cells of *zll* mutant embryos indistinguishably from wild-type (data not shown). This indicates that the *ZLL*-expressing cells were still present in the mutant and confirmed our histological observation that the morphology of the vascular system was not notably altered by mutations in the *ZLL* gene. In order to make sure that we only detected the *ZLL* transcript and not transcripts from related gene family members in *Arabidopsis*, we used three different regions of the *ZLL* gene as probes (see Materials and methods), all of which gave identical results in *in situ* hybridization experiments and did not result in cross-hybridization to genomic *Arabidopsis* DNA under low stringency hybridization conditions (data not shown).

Table II. Comparison of the floral organ numbers

Genotype	n	Organs			
		sepals	petals	stamens	carpels
wt	10	4.0±0.0	4.0±0.0	6.0±0.0	2.0±0.0
zll-3	25	4.2±0.4	4.2±0.5	5.8±0.3	2.2±0.3
clv1-4	34	5.0±0.9	5.5±0.8	8.0±1.2	5.5±1.3
zll-3 *clv1*	39	4.5±0.8	5.4±1.5	7.0±2.5	5.4±2.4

Mean numbers of floral organs and standard deviations are given.

Discussion

Our data address a critical process in shoot meristem development: the transition from early position-dependent activation of shoot meristem-specific genes in the embryo to a self-perpetuating stem cell system required for repetitive postembryonic organ formation. Mutations in the *ZLL* gene specifically disrupt embryonic shoot meristem development and result in the differentiation of stem cells.

Here we discuss the evidence that the primary defect in *zll* mutants is the failure to maintain central cells of the embryonic shoot meristem primordium in an undifferentiated state. We then address the critical events leading to the establishment of a self-perpetuating shoot meristem organization and the role of the *ZLL* gene in this process.

ZLL is required for shoot meristem cell fate in the embryo

The strongest defect observed in *zll* seedlings is a flat apex with cells that appear to have lost meristematic cell features and initiated differentiation. Therefore, we suggest that the primary defect in *zll* embryos is the failure to maintain apical cells in an undifferentiated state. In this view, ectopic cell differentiation and organ formation are secondary effects: being unspecified, the central cells are permitted to switch to an alternative developmental pathway. Differentiation of these cells may represent an intrinsic 'default' pathway, or be initiated in response to external cues.

The shoot meristem program appears to be initiated correctly in *zll* globular embryos. Later in embryogenesis, however, the cells in the center of the apex, which in wild-type constitute the stem cells, behave like peripheral cells in *zll*: they downregulate *STM* expression and initiate differentiation. After the initiation of the first true leaf primordia, the shoot meristem self-perpetuates independently of *ZLL*. The fact that *STM* is expressed during embryogenesis before *zll* development deviates from wild-type supports the notion that *stm* is epistatic to *zll* (Endrizzi *et al.*, 1996).

The *STM* gene is thought to prevent differentiation of central meristem cells (Long *et al.*, 1996). The *ZLL* gene appears to play a similar role that at least in part may be mediated by regulating the spatial expression pattern of *STM*. However, in contrast with *STM*, this role appears to be restricted to the developmental window between the torpedo embryo stage and the initiation of the first two leaf primordia. Expression of *STM* in lateral cells of the *zll* embryo apex may be a secondary effect, consistent with the notion that stem cells in the central zone of the wild-type shoot meristem suppress stem cell fate in neighboring regions (Loiseau, 1959).

The role of ZLL during postembryonic development

The embryo-specific role of *ZLL* in shoot meristem development contrasts with the roles of other genes that have been characterized in detail, such as the *CLV* genes (Clark *et al.*, 1997), *WUS* (Laux *et al.*, 1996) and *STM* (Clark *et al.*, 1996; Endrizzi *et al.*, 1996; Long *et al.*, 1996), each of which is continuously required for normal development of both shoot and floral meristems. Postembryonically, *zll* mutants can initiate and sustain functional adventitious shoot meristems, indicating that different processes establish the organization of primary (embryonic) and adventitious shoot meristems. The developing primary shoot meristem is surrounded by embryonic cells in which differentiation is initiated. Thus, it is conceivable that specific regulatory mechanisms may be required in the embryo to prevent the primary shoot meristem primordium from differentiating, and that *ZLL* is necessary for this regulation.

Molecular nature of ZLL

ZLL is a member of a novel group of related sequences that are highly conserved across the plant and animal kingdoms. There is more than one family member in a given organism. For example, nine *ZLL*-related putative genes are present in *C.elegans*. Only two *Arabidopsis* genes, *ZLL* and *AGO1*, have been genetically defined, but the sequences of the proteins do not suggest a molecular function. ZLL differs from related sequences at the N-terminus, suggesting that this region may be involved in a ZLL-specific function. In contrast, the high conservation at the C-terminus suggests that this region plays an important role for a general function of this gene family.

Expression of ZLL

ZLL mRNA was found in provascular cells and in the embryo apex. The transient expression of *ZLL* in the embryo apex matched the stage at which *zll* development deviates from wild-type. Thus, this *ZLL* expression in the embryo apex may promote stem cell fate in the center and/or restrict differentiation to the periphery of the shoot meristem primordium.

Alternatively, the expression in provascular cells may be crucial for shoot meristem development. In this view, the central–peripheral shoot meristem organization in the embryo transiently requires signaling from the vascular system to the apex that is promoted by ZLL. ZLL could mediate the transport of as yet unknown molecules, or might itself be transported to the embryo apex. A role for directed transport of plant hormones in embryonic shoot meristem establishment is conceivable (Fischer and Neuhaus, 1996). The specificity of the *zll* phenotype suggests that *ZLL* is essential only for embryonic shoot meristem formation, although it is expressed in provascular cells at all stages in development. One explanation is that other functions, possibly encoded by as yet unidentified *ZLL*-related *Arabidopsis* genes, can substitute for ZLL function in processes other than embryonic shoot meristem formation.

Role of ZLL

Our data suggest a role for *ZLL* in maintaining stem cells in an undifferentiated state during the transition from embryo-specific development to repetitive organ formation by a self-perpetuating shoot meristem. The similar phenotype of all *zll* alleles and the nature of the molecular lesions indicate that the mutants analyzed represent elimination, or at least a severe reduction, of ZLL activity.

Our results suggest that embryonic shoot meristem formation can be divided into two phases. Initially, apical cells in the globular embryo become specified as shoot meristem precursor cells and initiate expression of specific genes such as *STM*. Later in embryogenesis, the shoot meristem primordium becomes partitioned into a central zone harboring the stem cells and peripheral organ primordia. The initiation of the first true leaf primordia at the periphery coincides with the developmental time point whereafter the shoot meristem perpetuates independently of *ZLL*. Therefore, the central–peripheral partitioning of

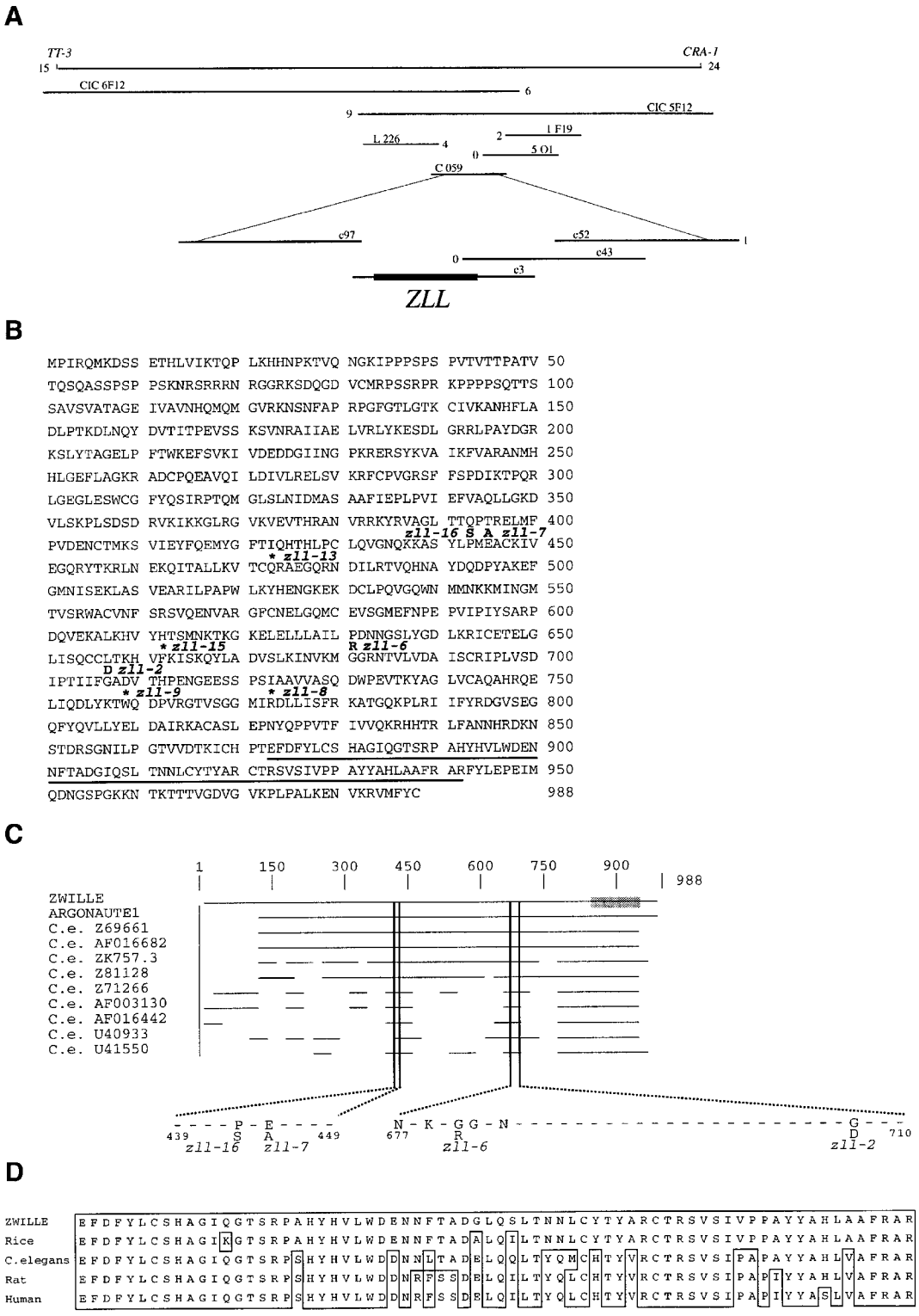

Fig. 6. Isolation of the *ZLL* gene and sequence comparison. (**A**) Map of genomic clones and location of the *ZLL* gene. The numbers of recombination breakpoints in 2500 meiotic events are given. (**B**) The ZLL protein sequence deduced from the longest reading frame of *ZLL* cDNA. The mutations in *zll*-13 and *zll*-9 result in stop codons (designated ★). The mutation in *zll*-15 causes a frame-shift, and the mutation in *zll*-8 changes an exon–intron border; both mutations result in predicted translational stops after a few codons (also designated ★). The mutations in *zll*-16, *zll*-7, *zll*-6 and *zll*-2 change the amino acid sequence. The most highly conserved region shown in (D) is underlined. (**C**) Schematic multiple alignment of ZLL, AGO1 and *C.elegans* sequences. Lines represent stretches of sequences with >37% similarity. Identical residues in all sequences compared are shown underneath. The missense mutations in *zll*-16, *zll*-7, *zll*-6 and *zll*-2 affect four of these residues. (**D**) Comparison of the ZLL protein region between residues 873 and 942 with sequences derived from the following sources: rice expressed sequence tag (EST; DDBJ/EMBL/GenBank accession no. C29031), *C.elegans* open reading frame (Wilson, 1994) (DDBJ/EMBL/GenBank accession no. Z69661), rat EST (DDBJ/EMBL/GenBank accession no. H31693) and human EST (DDBJ/EMBL/GenBank accession no. R91199). Residues identical to the ZLL sequence are shown in boxes.

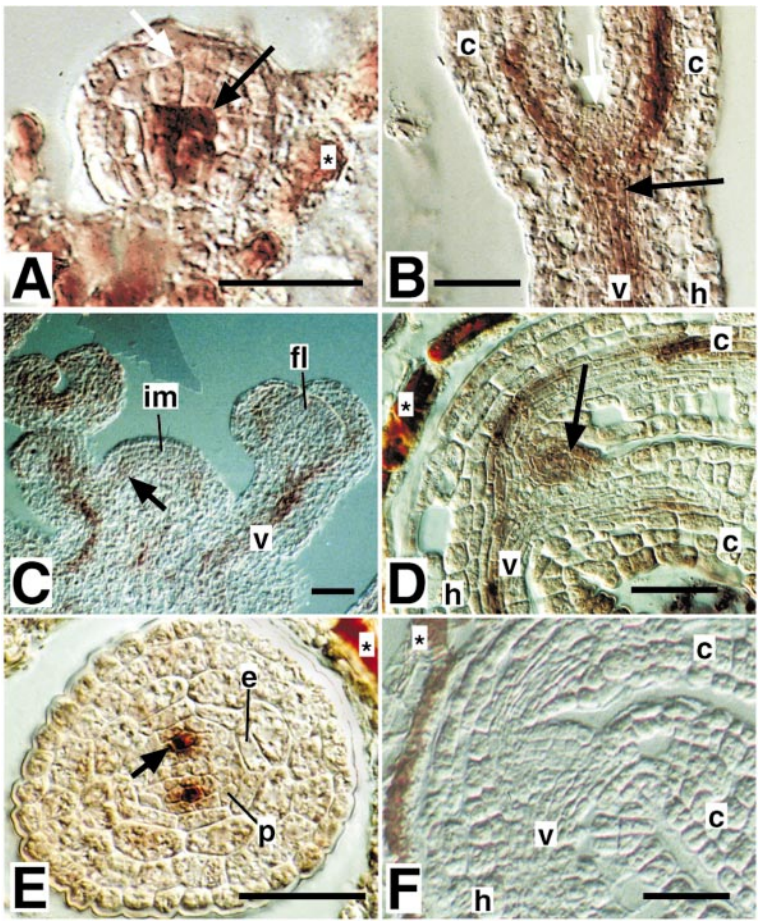

Fig. 7. *ZLL* gene expression pattern. *In situ* hybridization of *ZLL* antisense (A–E) and *ZLL* sense control RNA (F). In globular stage (**A**) and torpedo stage (**B**) embryos, *ZLL* is expressed in provascular cells (black arrow) but not in the embryo apex (white arrow). (**C**) In inflorescence, im, and floral, fl, meristems *ZLL* is expressed in provascular cells, apparently presaging the initiation of organs (arrow). (**D**) In bent cotyledon stage embryos *ZLL* is additionally expressed in the embryo apex (arrow). (**E**) Cross section of the embryo axis. *ZLL* expression is confined to what appear to be the phloem poles (arrow). (**F**) Sense control in a mature embryo. c, cotyledon; v, provascular cells; h, hypocotyl; e, endodermis; p, pericycle; ★, cells of the inner integument that show a brownish color independently of the *in situ* hybridization.

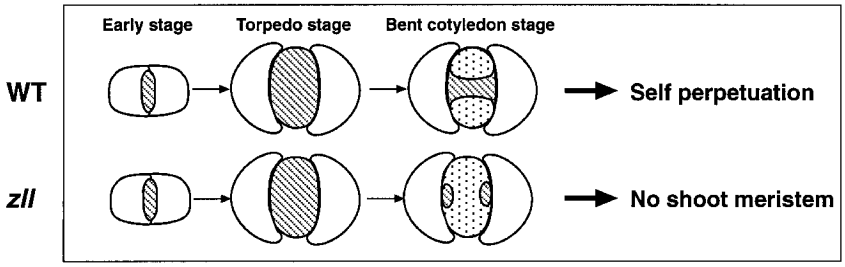

Fig. 8. The role of *ZLL*. Top view schematic comparison of wild-type (WT) and *zll* apical embryo development. Shoot meristem-specific genes are activated in cells (hatched) between the cotyledonary primordia (plain) at the globular stage in *zll* and wild-type. In early torpedo-stage embryos, the size of the expression domain has increased. Between the torpedo stage and the bent cotyledon stage, the apex in wild-type is partitioned into the first two leaf primordia at the periphery, where shoot meristem gene expression is discontinued and differentiation initiated (dotted), and the central zone harboring the stem cells. Subsequently, the shoot meristem self-perpetuates during postembryonic development. *zll* embryos fail to establish a wild-type central–peripheral organization and nearly all apical cells behave like peripheral wild-type cells, initiating differentiation.

the shoot meristem primordium might be a critical event to establish a self-perpetuating regulatory circuitry, involving genes like *STM* and *CLV1* (Clark *et al.*, 1996, 1997; Laux and Schoof, 1997).

The *ZLL* gene is required in this process, since in the absence of *ZLL* activity almost all apical cells, including those in the center, adopt peripheral fate: they discontinue meristem gene expression and initiate differentiation at the expense of the stem cells (Figure 8). Two alternative mechanisms for the role of *ZLL* are proposed: (i) *ZLL* prevents the differentiation of central cells within the shoot meristem primordium before central–peripheral partitioning has occurred. *ZLL* may prevent differentiation of stem cells in the center simply by maintaining meristem-specific gene expression in these cells until a self-perpetuating circuitry is established. (ii) Alternatively, *ZLL* may function in the partitioning of the embryo apex. In this view, *ZLL* is necessary to establish the boundary that

restricts differentiation to the periphery and allows the stem cells in the center to stay in an undifferentiated state. A similar mechanism has been proposed for *STM* function, and thus *ZLL* may affect partitioning by regulating the spatial *STM* expression pattern.

One important observation is that *zll*-related sequences are specific to multicellular organisms. Therefore, it is likely that *ZLL* functions in a regulatory process that is not specific to plants, but that is required generally to integrate individual cell fates into a multicellular context in both plants and animals. One conceivable model is that *ZLL* and related genes relay positional information, possibly by mediating cell–cell interactions. Further analysis of the molecular function of *ZLL* will provide insight into the regulation of integrative cell behavior, and this may aid understanding of the general role of members of this gene family in other organisms.

Materials and methods

The *zll*-1 allele was isolated in an independent mutagenesis experiment (Jürgens *et al.*, 1994). *zll*-2 to *zll*-16 were isolated in this work. *zll*-1 to *zll*-16 were analyzed after back crossing them three times. EMS mutagenesis, plant growth, crosses, electron scanning microscopy, histological sections and DAPI staining of nuclei were performed as described previously (Laux *et al.*, 1996).

Map-based cloning

YAC (Creusot *et al.*, 1995), BAC (L226, B122L226Q2; C059, B122C059Q2) and P1 clones (Liu *et al.*, 1995) were isolated and aligned to a contig by a series of hybridization experiments with end fragments. Recombination breakpoints were detected by RFLP analyses and PCR-based mapping. 15–20 kb fragments of the BAC clone C059, containing the *ZLL* gene, were subcloned into the vector pBINPLUS (van Engelen *et al.*, 1995). cDNA clones were isolated from a flower cDNA library (Weigel *et al.*, 1992) or a seedling cDNA library (Kieber *et al.*, 1993). To obtain mutant sequences, regions of the *ZLL* gene were amplified by PCR. The products of two independent PCRs were sequenced using nested primers. Genomic and cDNA clones were sequenced using the Sequenase Version 2.0 DNA Sequencing kit (USB) or the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham).

Hybridizations

In situ hybridization was performed using digoxigenin labeling as described (Jackson, 1991). Antisense RNA probes were made with T7 RNA-polymerase from the following regions of *ZLL* cDNA subcloned into the pBluescript vector (Stratagene): nt 47–552; nt 991–1687 and nt 1994–3323. Sense controls were synthesized from the same clones with T3 RNA-polymerase. Southern hybridization experiments were performed at 55°C in 6× SSC buffer following standard procedures.

Accession number

The *ZLL* sequence data has been submitted to the DDBJ/EMBL/GenBank database under accession number AJ223508.

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